

# Genetic Identification and Separation of Innate and Experience-Dependent Courtship Behaviors in *Drosophila*

Yufeng Pan<sup>1,\*</sup> and Bruce S. Baker<sup>1,\*</sup>

<sup>1</sup>Janelia Farm Research Campus, Howard Hughes Medical Institute, 19700 Helix Drive, Ashburn, VA 20147, USA

\*Correspondence: [pyfapf@gmail.com](mailto:pyfapf@gmail.com) (Y.P.), [bakerb@janelia.hhmi.org](mailto:bakerb@janelia.hhmi.org) (B.S.B.)

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## SUMMARY

Wild-type *D. melanogaster* males innately possess the ability to perform a multistep courtship ritual to conspecific females. The potential for this behavior is specified by the male-specific products of the *fruitless* (*fru<sup>M</sup>*) gene; males without *fru<sup>M</sup>* do not court females when held in isolation. We show that such *fru<sup>M</sup>* null males acquire the potential for courtship when grouped with other flies; they apparently learn to court flies with which they were grouped, irrespective of sex or species and retain this behavior for at least a week. The male-specific product of the *doublesex* gene (*dsx<sup>M</sup>*) is necessary and sufficient for the acquisition of the potential for such experience-dependent courtship. These results reveal a process that builds, via *dsx<sup>M</sup>* and social experience, the potential for a more flexible sexual behavior, which could be evolutionarily conserved as *dsx*-related genes that function in sexual development are found throughout the animal kingdom.

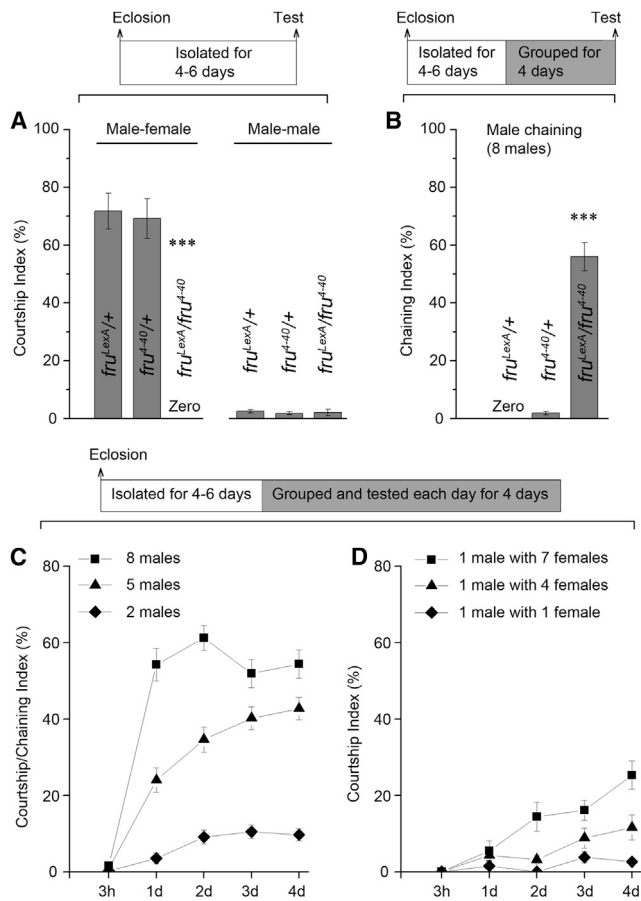
## INTRODUCTION

A fundamental goal of neuroscience is to elucidate how the neural circuits embodying the potential for particular behaviors are established. When this goal is considered from a neurogenetic perspective, a salient feature of behaviors is that they can be categorized as falling across a spectrum ranging from innate (i.e., built into the nervous system during development) to learned (i.e., acquired by experience) with many behaviors having some aspects that are innate and other aspects that are learned. Such a categorization of behaviors highlights two topics central to our findings. The first concerns whether there are commonalities as to how the potentials for innate behaviors and learned behaviors are acquired by the nervous system, or, alternatively, are they established by different mechanisms. Second, the past decade has seen a heightened interest in innate behaviors. This interest is, in part, because innate behaviors by their very nature are built into the nervous system during development, which makes it likely that they are (at some level) genetically specified. Thus genetically tractable animal species offer

the possibilities of identifying genes that specify innate behaviors and then employing these genes to generate reagents that can be used to probe issues related to this behavior, such as how the potential for that innate behavior is established in the nervous system (Baker et al., 2001).

Among innate behaviors, male courtship in *Drosophila melanogaster* is of particular interest because the genes responsible for establishing the potential for male courtship behavior have been identified (Baker et al., 2001; Ito et al., 1996; Ryner et al., 1996). These genes are the two terminal genes in the fly sex determination hierarchy, *fruitless* (*fru*) and *doublesex* (*dsx*), both of which encode sex-specific zinc finger transcription factors (Burtis and Baker, 1989; Ito et al., 1996; Ryner et al., 1996). The male-specific products of the P1 promoter of the *fru* gene (*fru<sup>M</sup>*) are expressed in a dispersed subset of ~2,000 neurons, which are found mostly in small groups throughout the central and peripheral nervous systems (Cachero et al., 2010; Lee et al., 2000; Manoli et al., 2005; Stockinger et al., 2005; Usui-Aoki et al., 2000; Yu et al., 2010). *fru<sup>M</sup>* function is both necessary and sufficient for building the potential for nearly all aspects of male courtship behavior into the nervous system (Demir and Dickson, 2005; Manoli et al., 2005, 2006). *dsx* encodes male- and female-specific DSX proteins (DSX<sup>M</sup> and DSX<sup>F</sup>, respectively) (Burtis and Baker, 1989), and DSX<sup>M</sup> is expressed in ~700 CNS neurons, the majority of which also express *fru<sup>M</sup>* (Billeter et al., 2006; Lee et al., 2002; Rideout et al., 2007, 2010; Robinett et al., 2010; Sanders and Arbeitman, 2008). DSX<sup>M</sup> is neither necessary nor sufficient for the execution of nearly all steps comprising courtship behavior (Taylor et al., 1994; Villella and Hall, 1996), but is required for one aspect of courtship song—sine song production (Rideout et al., 2007; Villella and Hall, 1996). In addition, in the absence of *dsx* function there is a poorly understood general diminution in the level of male courtship (Villella and Hall, 1996).

*fru<sup>M</sup>* functions postmitotically to endow the nervous system with the innate potential for male courtship behavior (Demir and Dickson, 2005; Lee et al., 2000; Manoli et al., 2005; Stockinger et al., 2005; Usui-Aoki et al., 2000). The gross neuroanatomical features of the *fru<sup>M</sup>*-expressing circuitry were previously found to be largely unaffected by the loss of *fru<sup>M</sup>*, as seen in *fru<sup>M</sup>* null males or wild-type females, leading to the conclusion that *fru<sup>M</sup>* largely functions to regulate fine neural connectivity or neural physiology (Cachero et al., 2010; Manoli et al., 2005; Stockinger et al., 2005). Indeed, imaging small groups of



**Figure 1. In the Absence of *fru<sup>M</sup>* Function *D. melanogaster* Males Acquire the Ability to Court as a Consequence of Being Group-Housed with Other Flies**

(A) Courtship indices of males that had been isolated since eclosion prior to a 10 min single-pair courtship test. \*\*\**p* < 0.001, one-way ANOVA.

(B) Chaining indices of groups of eight males that had been housed together for 4 days. \*\*\**p* < 0.001, one-way ANOVA.

(C) Chaining indices of groups of eight or five *fru<sup>LexA</sup>/fru<sup>4-40</sup>* males and courtship indices between 2 *fru<sup>LexA</sup>/fru<sup>4-40</sup>* males after grouping for 3 hr, 1 day, 2 days, 3 days, and 4 days.

(D) Courtship indices of *fru<sup>LexA</sup>/fru<sup>4-40</sup>* males to wild-type females after grouping (in groups of one male with seven females, one male with four females, or one male with one female) from 3 hr to 4 days. *n* = 8–12 for chaining behavior; *n* = 12–24 for others. *n* refers to number of pairs or groups, housing conditions are indicated in the top of each panel (apply to all figures). Error bars represent SEM.

See also Figure S1, Movies S1, and S2.

*fru<sup>M</sup>*-expressing neurons showed that the normal morphological development of many of these neurons requires *fru<sup>M</sup>* function (Cachero et al., 2010; Kimura et al., 2005, 2008; Lee and Hall, 2001; Mellert et al., 2010).

Although the proposal that *fru<sup>M</sup>* functions developmentally to specify the potential for male courtship is strongly supported by extant data, and has motivated and provided a framework for much of the recent work on courtship in *Drosophila* (Baker et al., 2001), there are features of the data on the role of *fru<sup>M</sup>* in courtship that suggest our understanding of the genetic specifi-

cation of *Drosophila* male courtship is significantly incomplete. In particular, courtship-like behaviors in the absence of *fru<sup>M</sup>* function have been reported (Anand et al., 2001; Shirangi et al., 2006; Vilella et al., 1997).

In the present study, we start by confirming the findings (Anand et al., 2001; Vilella et al., 1997) that males lacking *fru<sup>M</sup>* when housed together (group-housed) for a day or more during adulthood display courtship-like behaviors toward one another (male chaining). We then extend these observations to show that group-housing *fru<sup>M</sup>* null males with either conspecific females or females of other *Drosophila* species also leads to the acquisition by these males of the potential for courtship. In addition, we show that such *fru<sup>M</sup>*-independent, experience-dependent courtship behavior requires *fru<sup>M</sup>/dsx* overlapping neurons acutely during adulthood. Furthermore, *dsx<sup>M</sup>* is both necessary in *fru<sup>M</sup>* null males and sufficient in the otherwise wild-type females for the experience-dependent acquisition of the potential for courtship (with both conspecifics and other species). Finally, we show that *dsx<sup>M</sup>*- and experience-dependent courtship has properties indicative of learning and memory in that (1) the ability to court acquired via group-housing is retained for at least a week after being removed from that group, and (2) the sex and species of the flies *fru<sup>M</sup>* null males are group-housed with influence the male's courtship preferences in subsequent tests. Thus our findings uncover a *dsx<sup>M</sup>*- and experience-dependent pathway that is utilized by animals to acquire and modify courtship behavior based on their adult experiences. Some evolutionary implications of these findings are discussed.

## RESULTS

### Courtship Behavior of Isolated and Group-Housed *fru<sup>M</sup>* Null Males

For typical male-female courtship assays individual males are collected at eclosion and subsequently housed in isolation for 4–6 days prior to a single-pair (10 min) courtship test. With this protocol (1) wild-type control males intensively court virgin females (courtship index [CI] >60%, which is the fraction of observation time that males courted), but rarely court males, whereas (2) *fru<sup>M</sup>* null males (*fru<sup>LexA</sup>/fru<sup>4-40</sup>*) (Mellert et al., 2010) do not court females and only rarely court males (Figure 1A). These observations replicate previous findings that contributed to the proposal that wild-type *fru<sup>M</sup>* function specifies the potential for male courtship (Anand et al., 2001; Demir and Dickson, 2005; Ito et al., 1996; Manoli et al., 2005; Ryner et al., 1996).

In contrast, for assaying courtship-like interactions between multiple males, males are collected at eclosion, housed singly for 4–6 days, and then put together in groups of eight males for 4 days during which they were assayed daily for male-male courtship within the group (Anand et al., 2001; Vilella et al., 1997). With this protocol *fru<sup>M</sup>* null males showed intense courtship as quantified by a Chaining Index (ChI >50%, the fraction of time at least three males in the group engaged in courtship together) (Figure 1B; Movie S1 available online), a phenotype that has also been observed in other *fru<sup>M</sup>* null genotypes (Anand et al., 2001; Vilella et al., 1997). Groups of eight wild-type control

males that were treated identically did not exhibit chaining behavior (ChI ~0; Figure 1B).

Thus, there is a striking difference between the courtship behaviors displayed by *fru*<sup>M</sup> null males housed singly and tested in a pairwise courtship assay versus housed in groups and tested in a chaining assay. Comparing the protocols for these two behavioral assays suggests that the contrasting results could be caused by (1) housing conditions (housed singly before test versus housed in groups for 4 days before test), or (2) number of flies in a tested group (two versus eight), or (3) the target's sex (male versus female). The following experiments distinguish between these possibilities.

### Acquisition of Both Male-Male and Male-Female Courtship in *fru*<sup>M</sup> Null Males via Group-Housing

We first tested chaining behavior in groups of eight *fru*<sup>LexA</sup>/*fru*<sup>4-40</sup> males daily across the 4 days subsequent to grouping them together. Chaining behavior was observed at a low level following grouping for 3 hr (ChI[3h] = 1.6% ± 0.4%), and reached a high level after grouping for 1 or more days (ChIs[1d, 2d, 3d, 4d] >50%) (Figure 1C), consistent with previous findings (Anand et al., 2001; Villella et al., 1997). We then tested groups of either five or two *fru*<sup>M</sup> null males daily for 4 days and also found increased levels of chaining (five males) or male-male courtship (two males) as a function of time they were grouped (Figure 1C). These results suggest that it is the group-housing experience that induces chaining/courtship in *fru*<sup>M</sup> null males.

We next inquired whether prolonged housing of *fru*<sup>M</sup> null males with females also led to elevated levels of male-female courtship. We therefore placed single *fru*<sup>LexA</sup>/*fru*<sup>4-40</sup> males (4- to 6-day-old adults kept in isolation since eclosion) together with seven wild-type virgin females for 4 days during which male-female courtship was assayed daily. As expected, these *fru*<sup>M</sup> null males did not court females after 3 hr (CI = 0); however, these males very substantially increased their courtship to females after grouping for one or more days (CIs[1d, 2d, 3d, 4d] are 5.5% ± 2.6%, 14.4% ± 3.8%, 16.1% ± 2.6%, and 25.3% ± 3.7%, respectively) (Figure 1D; Movie S2). Increased courtship was also found when one male/four females and one male/one female combinations were housed together for varying periods of time prior to assaying courtship (Figure 1D). As male-male and male-female courtship behaviors (including chaining) were both observed in *fru*<sup>LexA</sup>/*fru*<sup>4-40</sup> males, these behaviors are hereafter collectively referred to as courtship.

Examining courtship by males of three other *fru*<sup>M</sup> null genotypes (*fru*<sup>LexA</sup>/*fru*<sup>sat15</sup>, *fru*<sup>4-40</sup>/*fru*<sup>sat15</sup>, *fru*<sup>4-40</sup>/*fru*<sup>AJ96u3</sup>, with the latter two genotypes lacking both *fru* P1 and P2 products) (Anand et al., 2001; Mellert et al., 2010; Song et al., 2002) after grouping from 3 hr to 4 days replicated the above findings with *fru*<sup>LexA</sup>/*fru*<sup>4-40</sup> males (Figure S1). These results establish that males without *fru*<sup>M</sup> function are able to court both male and female targets when group-housed for 1 or more days prior to testing.

### FRU<sup>M</sup>-Independent Courtship Behavior Requires the *fru*<sup>M</sup>/*dsx* Circuitry

As noted above, the male CNS contains ~2,000 *fru*<sup>M</sup>-expressing neurons as well as ~700 *dsx*-expressing neurons, with many

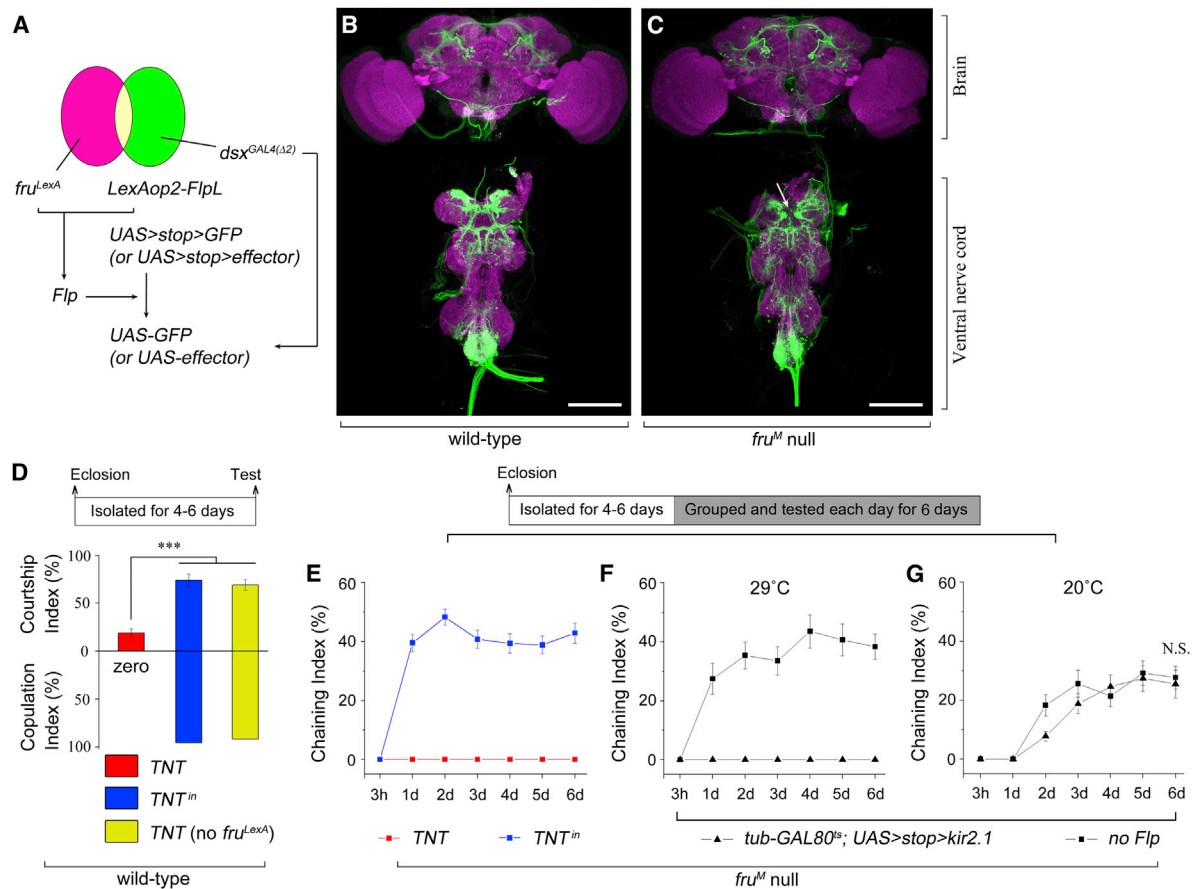
*dsx*-expressing neurons also expressing *fru*<sup>M</sup>. The morphology of *fru*<sup>M</sup> and *dsx* overlapping neurons in both the brain and ventral nerve cord (VNC), as specifically labeled by the intersection of *fru*<sup>LexA</sup> and *dsx*<sup>GAL4(Δ2)</sup> expression (Figure 2A), was not grossly different in males with or without FRU<sup>M</sup> function (Figures 2B and 2C, respectively), although detailed differences could be found, such as there is no midline crossing from gustatory receptor neurons, compared to males expressing FRU<sup>M</sup> (Mellert et al., 2010) (Figure 2C).

Given the presence of *fru*<sup>M</sup> and *dsx* overlapping neurons in both wild-type males and males lacking *fru*<sup>M</sup> function, we asked whether (some of) these neurons were required for *fru*<sup>M</sup>-independent courtship behavior.

In males that have FRU<sup>M</sup> function, when *fru*<sup>M</sup> and *dsx* overlapping neurons were silenced by expressing tetanus toxin light chain (TNT) (*LexAop2-FlpL/UAS* > *stop* > *TNT*; *dsx*<sup>GAL4(Δ2)</sup> *fru*<sup>LexA</sup>/+), courtship of females was severely impaired (CI ~20%), compared to control males expressing an inactive version of *TNT* (*TNT*<sup>in</sup>) in *fru*<sup>M</sup> and *dsx* overlapping neurons (*LexAop2-FlpL/UAS* > *stop* > *TNT*<sup>in</sup>; *dsx*<sup>GAL4(Δ2)</sup> *fru*<sup>LexA</sup>/+) or control males lacking the *fru*<sup>LexA</sup> driver (*LexAop2-FlpL/UAS* > *stop* > *TNT*<sup>in</sup>; *dsx*<sup>GAL4(Δ2)</sup>/+) (CIs ~70%) (Figure 2D). Furthermore, *TNT*-expressing males did not copulate with females (0 out of 24), while almost all control males copulated with females successfully (within 30 min) (Figure 2D). Thus, in males that have FRU<sup>M</sup> function, synaptic transmission from *fru*<sup>M</sup> and *dsx* overlapping neurons is necessary for robust courtship behavior of females and is also required for successful copulation.

When *TNT* was expressed in *fru*<sup>M</sup> and *dsx* overlapping neurons in *fru*<sup>M</sup> null males (*LexAop2-FlpL/UAS* > *stop* > *TNT*; *dsx*<sup>GAL4(Δ2)</sup> *fru*<sup>LexA</sup>/*fru*<sup>4-40</sup>), courtship was not observed even after 6 days of grouping (Figure 2E). Control *fru*<sup>M</sup> null males expressing *TNT*<sup>in</sup> in *fru*<sup>M</sup> and *dsx* overlapping neurons showed strong courtship after 1–6 days of grouping (ChIs ~40%) (Figure 2E).

To further test if the activity of *fru*<sup>M</sup> and *dsx* overlapping neurons is acutely required during adulthood for courtship by *fru*<sup>M</sup> null males, we used another neuronal silencer: the inward-rectifying potassium channel *Kir2.1* (Baines et al., 2001; Pfeiffer et al., 2010), together with a temperature-sensitive *GAL80*<sup>ts</sup> that blocks *Kir2.1* expression when flies are reared at lower temperature (18°C–20°C) but allows expression at 29°C. When *LexAop2-FlpL/tub-GAL80*<sup>ts</sup>; *dsx*<sup>GAL4(Δ2)</sup> *fru*<sup>LexA</sup>/UAS > *stop* > *Kir2.1 fru*<sup>4-40</sup> males were reared at 18°C, isolated after eclosion at 18°C for 4–6 days, heat shocked at 29°C for 1 day, then tested in groups of eight males for 6 days at 29°C, they did not show any courtship behavior. Control *fru*<sup>M</sup> null males lacking *LexAop2-FlpL* (*tub-GAL80*<sup>ts</sup>/+; *dsx*<sup>GAL4(Δ2)</sup> *fru*<sup>LexA</sup>/UAS > *stop* > *Kir2.1 fru*<sup>4-40</sup>) under the same conditions showed intensive courtship after grouping for 6 days (ChI ~40%; Figure 2F). When both experimental and control *fru*<sup>M</sup> null males were reared at 18°C, isolated after eclosion at 18°C for 5–7 days, then tested in groups of eight males for 6 days at 20°C, they performed courtship after 1–6 days grouping, although in slightly reduced levels (ChIs 20%–30%, Figure 2G), probably due to the lower temperature. These results indicate that activity of *fru*<sup>M</sup> and *dsx* overlapping neurons is necessary during adulthood for courtship acquisition in *fru*<sup>M</sup> null males.



**Figure 2. *fru<sup>M</sup>* and *dsx<sup>M</sup>* Overlapping Neurons Are Required for Both Wild-Type Courtship and *fru<sup>M</sup>*-Independent Courtship**

(A) A schematic of genetic strategy to label and manipulate *fru<sup>M</sup>* and *dsx<sup>M</sup>* overlapping neurons.

(B and C) Intersectional expression of *fru<sup>L,exA</sup>* and *dsx<sup>GAL4(Δ2)</sup>* in wild-type (B) and *fru<sup>M</sup>* null (C) backgrounds (genotypes: *LexAop2-FlpL/UAS > stop > myrGFP*; *dsx<sup>GAL4(Δ2)</sup> fru<sup>L,exA</sup>/+* and *LexAop2-FlpL/UAS > stop > myrGFP*; *dsx<sup>GAL4(Δ2)</sup> fru<sup>L,exA</sup>/fru<sup>4-40</sup>*, respectively). Arrow indicates no midline crossing by gustatory receptor neurons in *fru<sup>M</sup>* null background. Scale bars represent 100 μm.

(D) Courtship indices (upper) and copulation indices (lower) by males that have *fru<sup>M</sup>* function. \*\*\*p < 0.001, one-way ANOVA. n = 24 for each.

(E–G) Chaining indices by *fru<sup>M</sup>* null males after grouping from 3 hr to 6 days. n = 12 for each. Not significant (N.S.) by comparing chaining indices of day 6. Detailed genotypes are described in text. Error bars represent SEM.

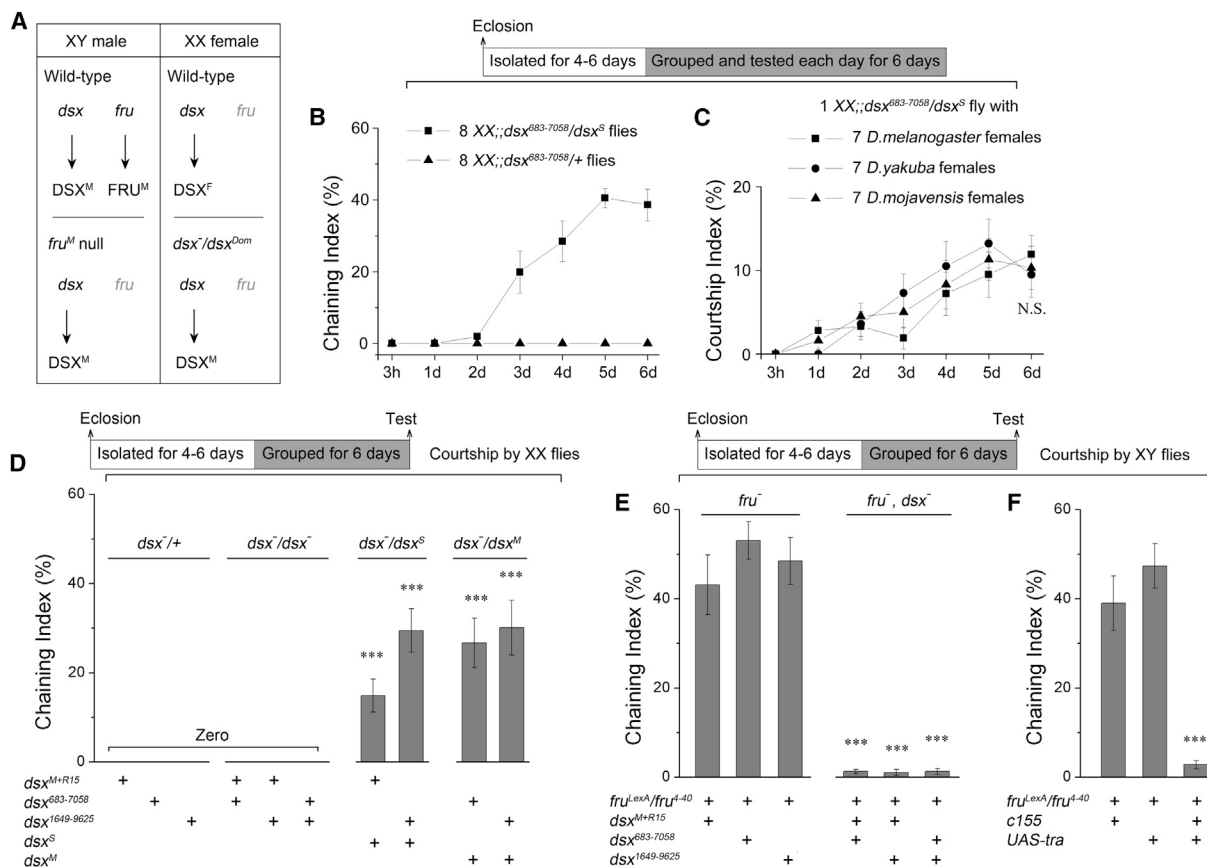
### ***dsx<sup>M</sup>* Is Both Necessary and Sufficient for *fru<sup>M</sup>*-Independent, Experience-Dependent Courtship**

That males lacking *FRU<sup>M</sup>* develop the potential for courtship when grouped, but wild-type females (that also lack *FRU<sup>M</sup>*) do not develop the potential for male courtship when grouped, suggests that non-*fru<sup>M</sup>*-specified sexual differences between males and females govern the ability to acquire the potential for *fru<sup>M</sup>*-independent courtship. If our understanding of the sex determination hierarchy is correct—that all aspects of somatic sex are controlled via the *fru<sup>M</sup>* and/or *dsx* branches of the hierarchy—then the ability to acquire male courtship behavior via group-housing must be governed by the *dsx* branch of the sex hierarchy. Thus chromosomal females (XX) that express *DSX<sup>M</sup>* in place of *DSX<sup>F</sup>*, but are otherwise wild-type, should be equivalent to chromosomal males (XY) that lack *FRU<sup>M</sup>* function but are otherwise wild-type (Figure 3A) and such females should be able to acquire the potential for male courtship behavior if grouped.

To test this prediction, we used *dsx* alleles, *dsx<sup>S</sup>*, *dsx<sup>M</sup>*, or *dsx<sup>D</sup>* (collectively termed *dsx<sup>Dom</sup>*), in which *dsx* pre-mRNA is constitutively spliced in the male pattern (Nagoshi and Baker, 1990), in combination with a *dsx* deficiency (either *Df(3R) dsx<sup>f00683-d07058</sup>*, *Df(3R) dsx<sup>f01649-d09625</sup>*, or *Df(3R) dsx<sup>M+R15</sup>*, hereafter referred to as *dsx<sup>683-7058</sup>*, *dsx<sup>1649-9625</sup>*, or *dsx<sup>M+R15</sup>*, respectively and collectively termed *dsx<sup>-</sup>*) (Chatterjee et al., 2011; Mellert et al., 2012), to generate XX; *dsx<sup>-</sup>/dsx<sup>Dom</sup>* individuals that express only the *DSX<sup>M</sup>* protein. The external sexual characteristics of these *dsx<sup>-</sup>/dsx<sup>Dom</sup>* females are indistinguishable from those of wild-type males (Baker and Ridge, 1980). When singly housed for 4–6 days posteclosion all XX; *dsx<sup>-</sup>/dsx<sup>Dom</sup>* combinations examined failed to court wild-type females in a 10 min single-pair courtship assay (data not shown), consistent with previous findings (Taylor et al., 1994).

We next grouped eight XX; *dsx<sup>683-7058</sup>/dsx<sup>S</sup>* flies together and examined behavior daily for 6 days. No courtship was observed 3 hr after grouping, but strikingly, these XX individuals showed





**Figure 3. *dsx<sup>M</sup>* Is Necessary and Sufficient in Both XX and XY Flies for *fru<sup>M</sup>*-Independent Courtship Behavior**

(A) Expression of sex-specific products of *fru<sup>M</sup>* and *dsx* in wild-type males and females, as well as in *fru<sup>M</sup>* null males and *dsx*-masculinized females.

(B) Chaining indices of groups of eight XX;*dsx<sup>683-7058</sup>/dsx<sup>S</sup>* flies after grouping from 3 hr to 6 days.

(C) Courtship indices of individual XX;*dsx<sup>683-7058</sup>/dsx<sup>S</sup>* flies to seven wild-type females or females of other species (*D. yakuba* or *D. mojavensis*). Not significant (N.S.) by comparing courtship indices of day 6.

(D) Chaining indices of groups of eight XX flies after 6-day grouping. \*\*\**p* < 0.001, compared with 0, one-sample *t* test.

(E and F) Chaining indices of groups of eight XY flies after 6-day grouping. \*\*\**p* < 0.001, one-way ANOVA. *n* = 8–12 for each. Genotypes indicated below. Error bars indicate SEM.

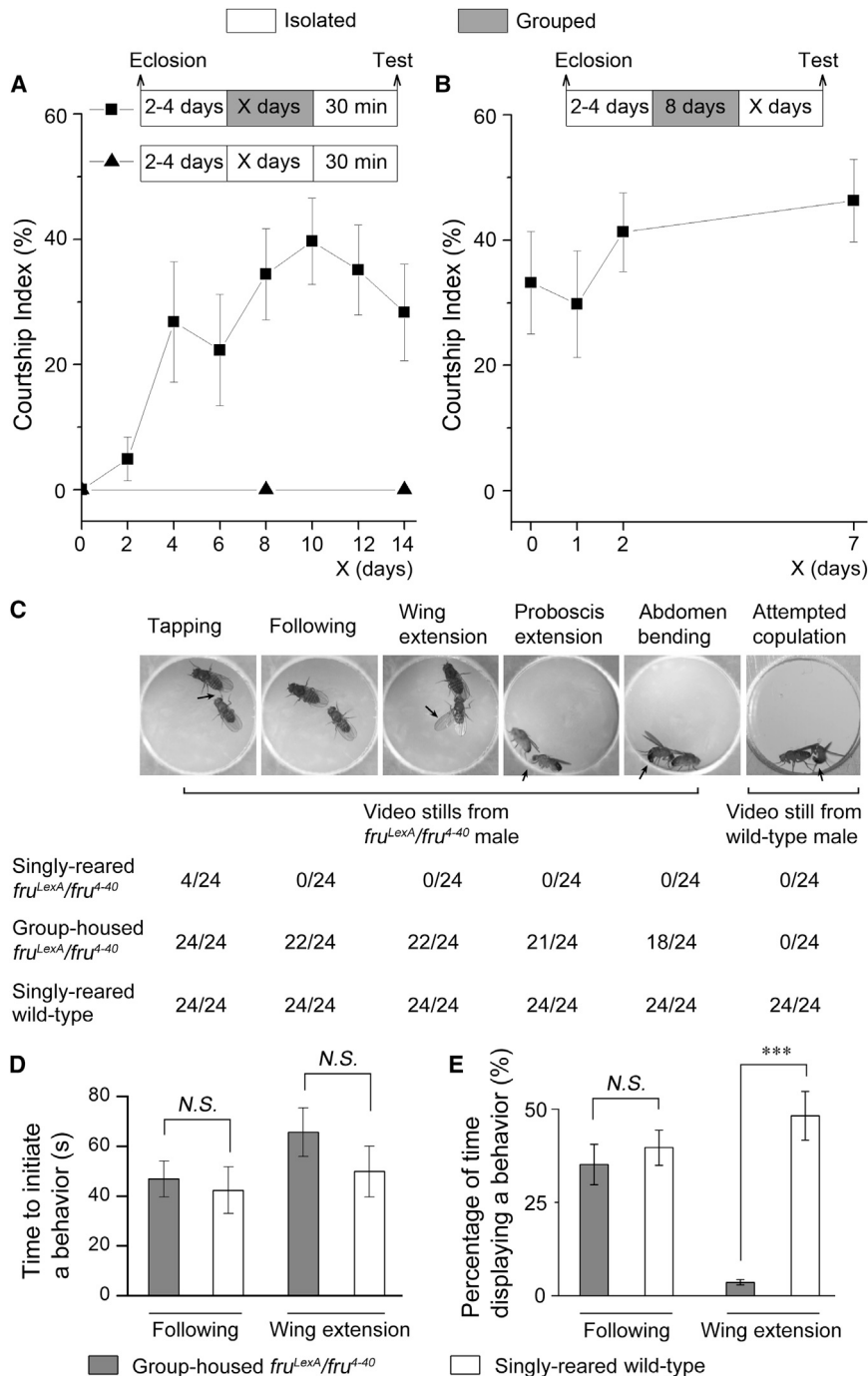
See also Movie S3.

substantial levels of courtship behavior after grouping for 1–6 days (Figure 3B; Movie S3). We then grouped 1 XX;*dsx<sup>683-7058</sup>/dsx<sup>S</sup>* fly with seven wild-type (*D. melanogaster*) females or females of other species (*D. yakuba* or *D. mojavensis*). Strikingly, these DSX<sup>M</sup>-expressing XX flies courted conspecific females as well as females of other species at similar levels when grouped for 1 or more days (Figure 3C). These results indicate that *dsx<sup>M</sup>* is sufficient in an otherwise wild-type female for courtship of both conspecific females or females of other species.

We further tested courtship in groups of eight XX individuals using four other *dsx<sup>-</sup>/dsx<sup>S</sup>* and *dsx<sup>-</sup>/dsx<sup>M</sup>* allelic combinations and observed significant courtship behavior in all four genotypes after grouping for 6 days (ChIs are 15%–30%, Figure 3D). XX individuals of two *dsx<sup>-</sup>/dsx<sup>D</sup>* combinations examined only rarely courted after 6 days grouping (ChIs ~1%, data not shown), which may be due to inefficiency of *dsx<sup>D</sup>* or its genetic background. As controls, XX individuals with either *dsx<sup>-</sup>/dsx<sup>-</sup>* or

*dsx<sup>-</sup>/+* combinations did not court after grouping for 6 days (Figure 3D). These experiments further demonstrate that in both XX and XY flies the expression of the DSX<sup>M</sup> protein (and the concomitant lack of the DSX<sup>F</sup> and FRU<sup>M</sup> proteins) are sufficient for the acquisition of the potential for male courtship as a consequence of group-housing.

To examine whether DSX<sup>M</sup> is necessary for acquiring the potential for FRU<sup>M</sup>-independent courtship, we group-housed XY individuals that lack both *fru<sup>M</sup>* and *dsx* functions and subsequently tested their ability to court. *fru<sup>M</sup>* and *dsx* double mutant males with three different allele combinations rarely courted even after grouping for 6 days (ChIs ~2%), while control *fru<sup>M</sup>* null males that express DSX<sup>M</sup> courted intensively after grouping for 6 days (ChIs ~50%) (Figure 3E). A caveat is that the above manipulations affect *dsx* activity in all tissues and thus could obscure its role in the CNS. Therefore we used a pan-neuronal *GAL4* driver (*c155*) to feminize (*UAS-tra*) only the neurons in *fru<sup>M</sup>* null males. Such *fru<sup>M</sup>* null males (*c155/Y*;



*UAS-tra/+; fru<sup>LexA</sup>/fru<sup>4-40</sup>*), which express *DSX<sup>F</sup>* in place of *DSX<sup>M</sup>* just in the nervous system, rarely courted even after grouping for 6 days (CI ~3%), while control *fru<sup>M</sup>* null males expressing *DSX<sup>M</sup>* courted intensively after grouping for 6 days (CIs ~40%), suggesting that *DSX<sup>M</sup>* function in the nervous system is necessary for the acquisition of the potential for courtship in *fru<sup>M</sup>* null males (Figure 3F). Thus *DSX<sup>M</sup>* is both necessary and sufficient for the *FRU<sup>M</sup>*-independent, experience-dependent courtship behavior.

fresh females after 2 days of grouping (CI is  $4.9\% \pm 3.5\%$ ) and such courtship increased and reached to its maximum after 10 days of grouping (CI is  $39.7\% \pm 6.9\%$ ) (Figure 4A). *fru<sup>M</sup>* null males kept in isolation for 10 or 16 days did not court females at all (Figure 4A). Thus the potential for courtship by *fru<sup>M</sup>* null males, which arises consequent to their grouping with females, represents a change intrinsic to those males.

To examine how long a *fru<sup>M</sup>* null male retained the ability to court after removal from a group of females, individual *fru<sup>M</sup>* null males

were first grouped with females for 8 days, then maintained in isolation for up to 7 days before testing courtship with a fresh female. We found that previously grouped *fru<sup>M</sup>* null males still intensively courted females after 7 days of isolation (CI is  $46.2\% \pm 6.6\%$ ) (Figure 4B), indicating that a quasi-permanent courtship ability was established via their group-housing experience with females.

We next asked whether mushroom bodies, which are involved in many forms of learning including courtship conditioning in wild-type males (Keene and Waddell, 2007; Vilella and Hall, 2008), are also required in the acquisition of the potential for male courtship in *fru<sup>M</sup>* null males. To do this, we first group-housed individual *fru<sup>M</sup>* null males that broadly express the temperature-sensitive *shibire<sup>ts1</sup>* (*shi<sup>ts1</sup>*) mutant (Kitamoto, 2001; Pfeiffer et al., 2012) in mushroom bodies (*UAS-shi<sup>ts1</sup>/+*; *19B03-GAL4 fru<sup>LexA</sup>/fru<sup>4-40</sup>* or *UAS-shi<sup>ts1</sup>/+*; *76D11-GAL4 fru<sup>LexA</sup>/fru<sup>4-40</sup>*, Figures S2A and S2B) (Jenett et al., 2012; Pfeiffer et al., 2008) with females at permissive temperature (23°C) for 8 days, then isolated those males for a subsequent courtship test with fresh females at either permissive (25°C) or restrictive (30°C) temperature and found that they courted intensively under both temperatures (Figure S2C), indicating that mushroom bodies are not required during courtship testing. We then silenced mushroom bodies in *fru<sup>M</sup>* null males by expressing *Kir2.1* (*tub-GAL80<sup>ts</sup>/+*; *19B03-GAL4 fru<sup>LexA</sup>/UAS-kir2.1::GFP fru<sup>4-40</sup>* or *tub-GAL80<sup>ts</sup>/+*; *76D11-GAL4 fru<sup>LexA</sup>/UAS-kir2.1::GFP fru<sup>4-40</sup>*) during adulthood including both group-housing and courtship testing and found that these males still courted intensively (Figure S2D), indicating that mushroom bodies are not necessary during either group-housing or testing for the acquisition and manifestation of courtship by *fru<sup>M</sup>* null males.

### Group-Housing Induces Most, but Not All, Steps of Courtship in *fru<sup>M</sup>* Null Males

Although previously group-housed *fru<sup>M</sup>* null males courted fresh females intensively, they did not copulate and were thus sterile. Analysis of their courtship showed that more than 75% of tested males performed most courtship steps including tapping, following, wing extension (no courtship song was detected, data not shown), licking (proboscis extension), and abdomen bending, but none of them attempted to copulate (by fully curling their abdomen toward a female's abdomen) (Figure 4C; Movie S4). These *fru<sup>M</sup>* null males initiated following and wing extension as quickly as wild-type males did (Figure 4D); however, they showed a much lower level of wing extension comparing to wild-type courtship (Figure 4E). As *fru<sup>M</sup>* null males were isolated 2–4 days prior to grouping with females, we further tested males that were isolated from 0 to 6 days before grouping but did not observe any difference in courtship, suggesting that there is no sensitive period for acquiring courtship during grouping. Thus, group-housing experiences induce most aspects of courtship in *fru<sup>M</sup>* null males, but some aspects (such as attempted copulation) are still missing and may be purely *fru<sup>M</sup>*-dependent.

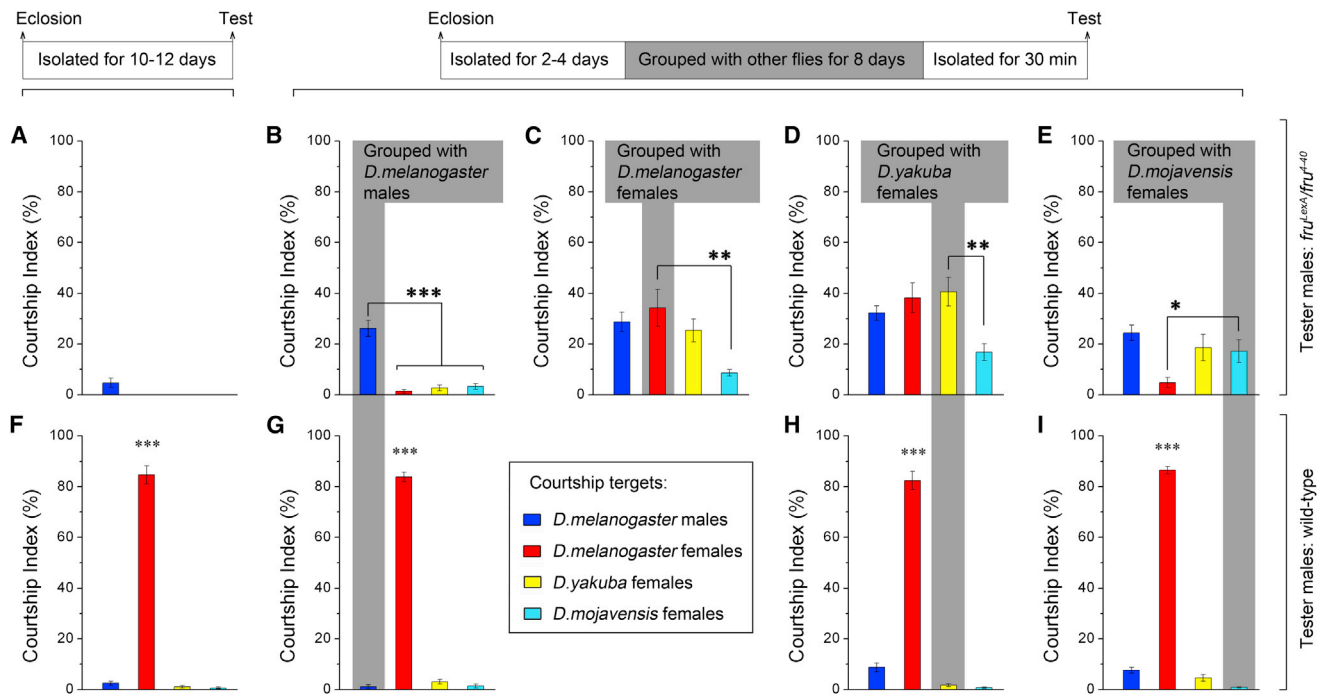
### Courtship Preference of Group-Housed *fru<sup>M</sup>* Null Males Depends on the Sex and Species of the Flies They Are Group-Housed With

The preceding experiments have established that housing *fru<sup>M</sup>* null *D. melanogaster* males together with groups of either

conspecific males or conspecific females leads to these *fru<sup>M</sup>* null males acquiring the potential to court the flies they have been housed with. Further, their acquisition of the ability to court is due to semipermanent change(s) that are intrinsic to these males. We can envision two broad types of mechanisms underlying the manifestation of these changes. First, these changes could be the consequences of an elevated level of general stimulation/excitation/arousal generated by social interactions. Alternatively, these changes could be the consequences of sensory perceptions provided by grouping having specific effects on the courtship circuitry (i.e., learning).

To distinguish between these possibilities we inquired whether grouping *fru<sup>M</sup>* null males with different courtship targets (conspecific *D. melanogaster* males and females and females of other species) would lead to differences in the quality of the courtship displayed by these males upon testing. Naive *fru<sup>M</sup>* null males that had been isolated until the courtship test only rarely displayed courtship between two such males and did not court *D. melanogaster*, *D. yakuba*, or *D. mojavensis* females (Figure 5A). Control wild-type *D. melanogaster* males, regardless of whether isolated or grouped prior to a courtship test, all courted conspecific females intensively, but rarely courted conspecific males or females of other species (Figures 5F–5I). When *fru<sup>M</sup>* null males were housed together in food vials (11 males per vial) for 8 days and then isolated for a subsequent courtship test, two such males courted one another intensively (CI is  $26.2\% \pm 3.1\%$ ), but rarely courted either conspecific females or females of other species (Figure 5B). In contrast, when individual *fru<sup>M</sup>* null males were housed with ten *D. melanogaster* females in food vials for 8 days and then isolated for a subsequent courtship test, they courted *D. melanogaster* male and female targets intensively (CI<sub>male-male</sub> is  $28.7\% \pm 3.9\%$  and CI<sub>male-female</sub> is  $34.3\% \pm 7.3\%$ ) and *D. yakuba* females at a similar level (CI is  $25.4\% \pm 4.5\%$ ), but *D. mojavensis* females at a reduced level (CI is  $8.6\% \pm 1.3\%$ ) (Figure 5C). We further found that *fru<sup>M</sup>* null males that had been grouped with *D. yakuba* females courted *D. yakuba* females, as well as *D. melanogaster* males and females at similar levels, but *D. mojavensis* females at a reduced level (Figure 5D). Strikingly, *fru<sup>M</sup>* null males that had been grouped with *D. mojavensis* females courted females of *D. mojavensis* and *D. yakuba*, as well as *D. melanogaster* males at similar levels, but they courted conspecific *D. melanogaster* females at a reduced level (Figure 5E). These findings demonstrate that the quality of a *fru<sup>M</sup>* null male's grouping experience determines at least some features of the courtship potential he acquires and thus favor the proposition that group-housing experiences have specific learning effects on the courtship circuitry in *fru<sup>M</sup>* null males.

Our results indicate that courtship by males that lack *fru<sup>M</sup>* function is, at least in part learned, as their mate preference is significantly dependent on prior experiences; while courtship by males that have *fru<sup>M</sup>* function is innate, and their mate choice is not significantly modifiable by our manipulations, suggesting that *fru<sup>M</sup>* may function to suppress *dsx<sup>M</sup>*-dependent courtship to inappropriate targets.



**Figure 5. Flexibility of the Grouping-Induced Courtship Behavior in *fru<sup>M</sup>* Null Males**

All males were tested either between two males of the same genotype (blue), toward *D. melanogaster* females (red), *D. yakuba* females (yellow), or *D. mojavensis* females (cyan).

(A–E) Courtship indices of *fru<sup>LexA</sup>/fru<sup>4-40</sup>* males that had been isolated (A), or grouped with males of the same genotype (B), *D. melanogaster* females (C), *D. yakuba* females (D), or *D. mojavensis* females (E) for 8 days prior to testing. \**p* < 0.05, \*\**p* < 0.01 and \*\*\**p* < 0.001, one-way ANOVA.

(F–I) Courtship indices of wild-type males that had been isolated (F), or grouped with males of the same genotype (G), *D. yakuba* females (H), or *D. mojavensis* females (I) for 8 days prior to testing. \*\*\**p* < 0.001, one-way ANOVA. *n* = 12–24 for each. Error bars represent SEM.

### Contributions of Sensory Stimulation to the Acquisition of Courtship Behavior in *fru<sup>M</sup>* Null Males

The above data suggest that courtship by *fru<sup>M</sup>* null males is dependent on sensory perceptions during both (1) a multiday grouping period that is crucial for courtship acquisition, and (2) a 10 min courtship test period. Here, we present our initial experiments to examine the roles of individual sensory modalities in the acquisition and manifestation of courtship behavior in *fru<sup>M</sup>* null males.

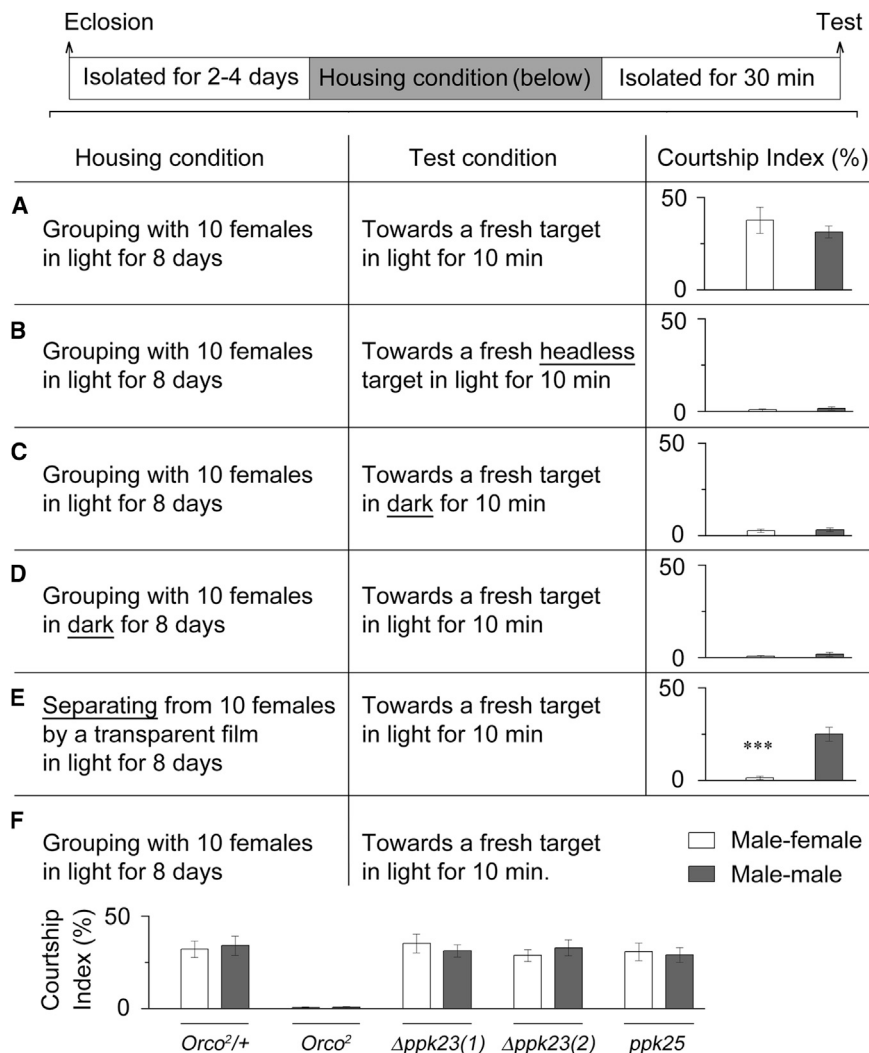
We first examined the role(s) of vision during group-housing and the courtship test. When individual *fru<sup>LexA</sup>/fru<sup>4-40</sup>* males were group-housed with ten wild-type females in food vials with constant light for 8 days, then isolated for a subsequent courtship test with intact targets in the light, such males courted both male and female targets intensively (Figures 5C and 6A). When such males were tested with headless (largely stationary) targets, or intact targets in the dark, they rarely courted either male or female targets (Figures 6B and 6C), suggesting that visual information (especially motion detection) is required during testing for courtship by *fru<sup>M</sup>* null males, consistent with previous findings with respect to *fru<sup>M</sup>* null males with all *dsx*-expressing neurons activated (Pan et al., 2011). When individual *fru<sup>LexA</sup>/fru<sup>4-40</sup>* males were group-housed with ten wild-type females in food vials under constant dark condition for 8 days, then isolated for a subsequent courtship test with intact targets in the light, they did not court either male or female targets (Fig-

ures 6D), indicating that vision during group-housing is required for the acquisition of the potential for courtship behavior by *fru<sup>M</sup>* null males.

To further explore the role(s) of vision in these processes we next used two-layered behavioral chambers in which the top and bottom halves of the chamber were separated by a transparent plastic sheet. The top half of the chamber housed individual *fru<sup>LexA</sup>/fru<sup>4-40</sup>* males who were provided with a dollop of food at the edge of the chamber, while the bottom layer of the chamber housed ten wild-type females on food. Flies were kept in these split chambers under constant light for 8 days (to allow only visual stimulation from females), and the males were then extracted for a subsequent courtship test with an intact target in the light. Surprisingly, these *fru<sup>M</sup>* null males courted males intensively, but rarely courted females (Figure 6E), suggesting that visual stimulation from females alone during group-housing is sufficient to induce the potential for male-male courtship behavior, while nonvisual stimulation during group-housing may be required for the acquisition of the potential for male-female courtship.

We then tested the role of olfactory perception in eliciting the potential of courtship in *fru<sup>M</sup>* null males. The *Orco<sup>2</sup>* mutant allele of the broadly expressed olfactory receptor gene *Orco* (aka *Or83b*) (Larsson et al., 2004) was introduced into a *fru<sup>M</sup>* null background (*Orco<sup>2</sup> fru<sup>LexA</sup>/Orco<sup>2</sup> fru<sup>4-40</sup>*), and such individual males were group-housed with ten wild-type





**Figure 6. Sensory Basis of the Grouping-Induced Courtship Behavior in *fru*<sup>M</sup> Null Males**

(A–E) Courtship indices by *fru*<sup>LexA</sup>/*fru*<sup>4-40</sup> males with male (gray) or female (white) targets. Manipulations of visual inputs during group-housing and/or courtship testing are described (highlighted by underlines). \*\*\*p < 0.001, two-sample t test.

(F) Courtship indices by *fru*<sup>LexA</sup>/*fru*<sup>4-40</sup> males with *Orco*, *ppk23*, or *ppk25* mutations. Detailed genotypes are described in text. n = 12–24 for each. Error bars represent SEM.

Please see Figure S2.

We further tested the role of gustatory receptor genes *ppk23* and *ppk25*, which have been recently reported to be involved in wild-type male courtship behavior (Lu et al., 2012; Starostina et al., 2012; Thistle et al., 2012; Toda et al., 2012), in the group-housing induced courtship by *fru*<sup>M</sup> null males, but did not observe any defect in these mutants in a *fru*<sup>M</sup> null background (genotypes are:  $\Delta ppk23(1)/Y$ ; *fru*<sup>LexA</sup>/*fru*<sup>4-40</sup>,  $\Delta ppk23(2)/Y$ ; *fru*<sup>LexA</sup>/*fru*<sup>4-40</sup> and *ppk25*( $\Delta 5-2$ )/*ppk25*( $\Delta 5-22$ ); *fru*<sup>LexA</sup>/*fru*<sup>4-40</sup>) (Figure 6F). Consistent with this, silencing *ppk23*-expressing or *ppk25*-expressing neurons in *fru*<sup>M</sup> null males (genotypes are: *UAS-shi*<sup>ts1</sup>/+; *ppk23*-GAL4(1) *fru*<sup>LexA</sup>/*fru*<sup>4-40</sup>, *UAS-shi*<sup>ts1</sup>/*ppk23*-GAL4(2); *fru*<sup>LexA</sup>/*fru*<sup>4-40</sup> and *UAS-shi*<sup>ts1</sup>/*ppk25*-GAL4; *fru*<sup>LexA</sup>/*fru*<sup>4-40</sup>) during just the test phase did not affect courtship behavior (Figure S2C). However, these results do not exclude the possible

involvement of gustatory perception provided by other receptor genes in courtship by *fru*<sup>M</sup> null males.

females in food vials in constant light for 8 days, then isolated for a subsequent courtship test with an intact target in the light. These males failed to court either male or female targets, while control males (*Orco*<sup>2</sup> *fru*<sup>LexA</sup>/*fru*<sup>4-40</sup>) courted both male and female targets (Figure 6F) (similar findings in control *fru*<sup>LexA</sup>/*Orco*<sup>2</sup> *fru*<sup>4-40</sup> males, data not shown). These results indicate that olfactory perception is necessary during group-housing or courtship test, or in both phases, for courtship acquisition and manifestation in *fru*<sup>M</sup> null males. Silencing *Orco*-expressing neurons during just the test phase impaired courtship by *fru*<sup>M</sup> null males (*UAS-shi*<sup>ts1</sup>/*Orco*-GAL4; *fru*<sup>LexA</sup>/*fru*<sup>4-40</sup>) toward either male or female targets (Figure S2C; data not shown), indicating that olfactory perception is indeed necessary for courtship during testing. Taken together with the above results using split chambers, our results show that for male-male courtship, olfactory input is necessary during the test phase, but not necessary during group-housing phase if visual input is provided; for male-female courtship, olfactory input may be necessary in both group-housing phase and test phase.

involvement of gustatory perception provided by other receptor genes in courtship by *fru*<sup>M</sup> null males.

## DISCUSSION

For nearly 100 years male courtship behavior in *D. melanogaster* has been recognized as a robust, complex, and largely innate behavior: a male fly is fully capable of performing all steps of courtship behavior when raised in complete isolation from egg to adulthood and then presented with a female fly as his first encounter with another creature. Thus male courtship has been used as a model system for the analysis of such topics as, how innate behaviors are elicited by specific environmental cues and how sequential motor programs are coordinated (Baker et al., 2001; Greenspan and Ferveur, 2000).

One of the most significant findings with respect to courtship behavior during the last decade is that a single gene (*fru*<sup>M</sup>) is both necessary and sufficient for building the potential of courtship behavior into a dedicated courtship circuitry (Demir and Dickson, 2005; Manoli et al., 2005, 2006; Stockinger et al.,

2005). Here, we show that while courtship behavior is abolished in *fru<sup>M</sup>* null males that are raised in isolation, a condition used by most studies in this field, many steps of courtship behavior can be alternatively established simply by group-housing *fru<sup>M</sup>* null males with either male or female flies for 1 or more days prior to testing. We further demonstrated that such *fru<sup>M</sup>*-independent, experience-dependent courtship is genetically specified by the *dsx* gene, whose expression significantly overlaps that of *fru<sup>M</sup>* in the CNS. Finally, we show that the experience-dependent acquisition of the potential for courtship has properties indicative of learning and memory, but is independent of mushroom bodies. Integrating our results with previous findings deepens our understanding of both the genetic and neuronal underpinnings of courtship.

Numerous studies have contributed to identifying *fru<sup>M</sup>* as a dedicated regulatory gene that specifies the neural substrates of *D. melanogaster* male courtship (Demir and Dickson, 2005; Ito et al., 1996; Manoli et al., 2005; Ryner et al., 1996; Stockinger et al., 2005) and showing that *fru<sup>M</sup>* largely functions to regulate fine neural connectivity and/or neural physiology (Cachero et al., 2010; Kimura et al., 2005, 2008; Manoli et al., 2005; Mellert et al., 2010; Stockinger et al., 2005). More recent findings have highlighted the importance of *dsx*-expressing neurons, and in particular those that also express *fru<sup>M</sup>*, in male courtship (Kimura et al., 2008; Pan et al., 2011, 2012; Rideout et al., 2007). Of particular relevance, in the light of our discovery of a *fru<sup>M</sup>*-independent, *dsx*-dependent, and experience-dependent courtship pathway, is the finding that artificial activation of all *dsx* neurons elicits courtship by males independent of whether they had functional *fru<sup>M</sup>* (Pan et al., 2011). Approximately two-thirds of all *dsx*-expressing CNS neurons are found in the ventral nerve cord, and in particular the abdominal ganglion, where they likely function in the execution of sexual behaviors. This leaves five bilaterally present clusters of *dsx*-expressing neurons in the brain (~300 neurons total counting both hemispheres) as likely containing the *dsx* neurons that mediate the acquisition of experience-dependent courtship. Of these five clusters of *dsx*-expressing neurons, the male-specific PC1 (also termed P1) cluster, which expresses both *fru<sup>M</sup>* and *dsx<sup>M</sup>*, is a particularly attractive candidate for having a significant role in experience-dependent courtship, based on its key role in initiating *fru<sup>M</sup>*-dependent courtship (Kimura et al., 2008; Kohatsu et al., 2011; Pan et al., 2012).

Our findings add significantly to understanding the role of *dsx<sup>M</sup>* in specifying male courtship behavior. Previous studies showed that in males that are wild-type for *fru<sup>M</sup>* one specific aspect of male courtship—sine song—is dependent on *dsx<sup>M</sup>* function (Villega and Hall, 1996). Thus, it is likely that the potential for sine song is innately established in CNS by *dsx<sup>M</sup>* in a manner analogous to how the potential for *fru<sup>M</sup>*-dependent aspects of courtship are innately established. Additionally, in *dsx* null males that are wild-type for *fru<sup>M</sup>* there is a poorly understood deficit in the overall level of courtship (as measured by the CI), but all steps of courtship occur, except for sine song and copulation itself, which is mechanically not possible due to *dsx*-dependent defects in genital development. Our results reveal additional roles of *dsx<sup>M</sup>* in the acquisition of the potential for courtship in the absence of *fru<sup>M</sup>* function. This reasoning suggests that *dsx<sup>M</sup>* functions both to facilitate acquisition of the potential for

many aspects of courtship (in the absence of *fru<sup>M</sup>*) and to (in the presence of *fru<sup>M</sup>*) innately determine at least one aspect of courtship—sine song.

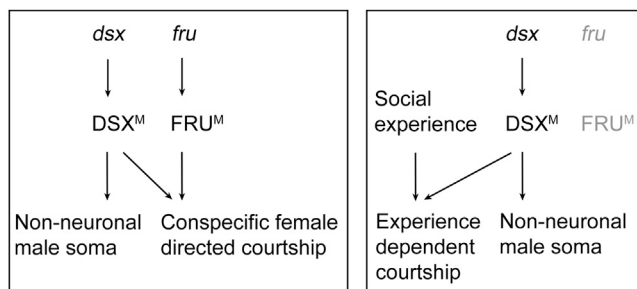
### Specification of Courtship Circuitry during Development and in Adults

As we noted above, the sex determination genes *fru<sup>M</sup>* and *dsx<sup>M</sup>* in males function developmentally to build some aspects of courtship behavior into the CNS. Although the majority of neurons comprising the courtship circuitry are still present in *fru<sup>M</sup>* null males, they do not function effectively in transducing sensory cues to motor centers that execute courtship behavior. Strikingly, group-housing experience allows efficient transduction from sensory cues to motor centers when *fru<sup>M</sup>* is not expressed. Thus social experience acting via *dsx<sup>M</sup>*-mediated processes somehow compensates for many aspects of *fru<sup>M</sup>* function. We note that other aspects of courtship behavior (e.g., attempted copulation) are not observed in *fru<sup>M</sup>* null males, even after they have been group-housed, suggesting that the latter aspects of courtship are solely *fru<sup>M</sup>*-dependent.

How does social experience change the courtship circuitry in the absence of *fru<sup>M</sup>*? We note that many recent studies on flies have found that social experience can change gene expression, synaptic connectivity, and/or pheromone profiles (Bushey et al., 2011; Carney, 2007; Donlea et al., 2009, 2011; Farine et al., 2012; Krupp et al., 2008). As our study showed that when *fru<sup>M</sup>* null males that had been group housed were isolated and then singly housed for 7 days, they still courted fresh females intensively, it is unlikely that changed pheromone profiles, if any, play essential roles in establishing courtship behavior in *fru<sup>M</sup>* null males. Rather, we suggest that social experience induces courtship in *fru<sup>M</sup>* null males by changing gene expression and/or neuronal connectivity to allow efficient transduction from sensory perception to motor output. Whether social experience functioning through *dsx<sup>M</sup>* during adulthood and *fru<sup>M</sup>* functioning during development, act through identical or synonymous mechanisms to specify the courtship circuitry is unknown and awaits further study. In this regard, we note that the experience-dependent acquisition of the potential for male courtship behavior during adulthood provides a robust single fly paradigm for learning that may facilitate studies of learning at a variety of levels.

### An Evolutionary Scenario

That two alternative systems for establishing the potential for male courtship coexist in *D. melanogaster* raises the question of why it is genetically structured in this way (Figure 7). One possibility is that *fru<sup>M</sup>*'s specification of innate courtship in *D. melanogaster* represents a system that has evolved from an ancestral state in which the potential for courtship was acquired through social interactions and *dsx*. It has been hypothesized that learned behavioral adaptations evolutionarily precede innately specified forms of the same behaviors (Tierney, 1986). That *fru<sup>M</sup>* has not been identified outside of insects, whereas *dsx*-related genes (DMRTs) that function in sexual development are found throughout the animal kingdom (Kopp, 2012) is consistent with this view. We also note that, because DMRTs are deeply conserved, the *dsx<sup>M</sup>*/social experience pathway may be



**Figure 7. Alternative Modes of Specifying Male Courtship in *Drosophila***

As *dsx* is expressed in both nonneuronal somatic cells as well as neurons, we distinguish its role in the nervous system for male courtship from nonneuronal somatic development. In contrast, *fru*<sup>M</sup> is expressed exclusively in neurons. Left: FRU<sup>M</sup> and DSX<sup>M</sup> jointly specify wild-type courtship that is conspecific female directed. Right: in the absence of FRU<sup>M</sup>, social experience and DSX<sup>M</sup> jointly specify the experience-dependent courtship that is more flexible.

functional in other species, including those such as humans that show more flexibility in their courtship rituals.

## EXPERIMENTAL PROCEDURES

### Fly Stocks

*fru*<sup>M</sup> null alleles used in this study include *fru*<sup>LexA</sup>, *fru*<sup>4-40</sup>, *fru*<sup>sat15</sup>, and *fru*<sup>AJ96u3</sup>. *dsx* null alleles are *dsx*<sup>683-7058</sup>, *dsx*<sup>1649-9625</sup>, and *dsx*<sup>M+R15</sup>. *dsx* dominant alleles are *dsx*<sup>S</sup>, *dsx*<sup>M</sup>, and *dsx*<sup>D</sup>. *dsx*<sup>GAL4(Δ2)</sup> was described in Pan et al. (2011). UAS > stop > TNT and UAS > stop > TNT<sup>tn</sup> were provided by B. Dickson. *LexAop2-FlpL*, UAS > stop > myrGFP, UAS > stop > Kir2.1, UAS-*shi*<sup>ts1</sup>, UAS-*kir2.1::GFP*, 19B03-GAL4, and 76D11-GAL4 were gifts from G. Rubin. *Δppk23* and *ppk23-GAL4* lines were obtained independently from K. Scott (referred to as *Δppk23(1)* and *ppk23-GAL4(1)*) and Y. Ben-Shahar (referred to as *Δppk23(2)* and *ppk23-GAL4(2)*). *ppk25(Δ5-2)*, *ppk25(Δ5-22)*, and *ppk25-GAL4* were gifts from C. Pikielny. All crosses were performed and kept at room temperature (~23°C), unless stated otherwise.

### Courtship Assays

Two courtship assays were used: (1) for assaying a one-time single-pair courtship (Figures 1A, 2D, 4, 5, 6, and S2), small round two-layer chambers (diameter: 1 cm; height: 2.5 mm per layer) were used. Individual tester flies and target flies were gently aspirated into different layers and were separated by a plastic transparent barrier that was removed ~30 min later to allow the courtship test; (2) for assaying chaining/courtship behavior of multiple flies (Figures 1B–1D, 2E–2G, 3, and S1), large round one-layer chambers were used (diameter: 4 cm; height: 3 mm). Groups of tester flies were briefly cooled on ice and loaded into the chamber. Tests were performed daily for up to 6 days (3 hr after grouping as day 0, then days 1–6). For both assays, tests were performed at 25°C on fly food that was at the bottom of behavioral chambers, unless stated otherwise. Each test was performed for 10 min. For detailed preparation of courtship tester and target flies, see the Supplemental Information.

### Analysis of Courtship Behavior

Courtship index (CI), which is the percentage of observation time a fly performs any courtship step (Vilella et al., 1997), was used to measure courtship to female targets or between two males. In the case where there are multiple female targets (such as one male and seven females in Figure 1D), CI represents courtship to all targets. Paired male-male courtship used two males of the same genotype, but focused on the male fly that first initiated courtship (courtship of the initiator to the other). Chaining index (ChI), which is the percentage of observation time at least three flies engaged in courtship together, was used to measure courtship in groups of eight flies (and groups of five flies

in Figure 1C). Comparison of two indices was made by two-sample t test, and comparison between multiple groups was made by one-way ANOVA.

### Tissue Dissection, Staining, and Imaging

CNSs were dissected at 4–6 days posteclosion (Figures 2A and 2B), unless stated otherwise (Figure S2E). Antibodies used were rabbit anti-GFP (Invitrogen A11122) 1:1,000, mouse anti-Bruchpilot (Developmental Studies Hybridoma Bank nc82) 1:30, and secondary Alexa Fluor 488 and 568 antibodies (1:500). Samples were imaged at 20× magnification on a Zeiss 710 confocal microscope and processed with Fiji software.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, two figures, and five movies and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2013.11.041>.

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